COMMENTS ON THE NATIONAL TOXICOLOGY PROGRAMME (NTP) DRAFT REPORT ON CARCINOGENS: BACKGROUND DOCUMENT ON GLASS WOOL FIBRES

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I have been asked by the European Insulation Manufacturers Association (EURIMA) to review and provide comments on the "Draft report on carcinogens: background document on glass wool fibres". My comments are confined to addressing the utility of *in vitro* tests to classify fibres as carcinogenic, as this is my own special field of expertise.

Qualifications

I am Professor of Respiratory Toxicology at the University of Edinburgh and an independent consultant on pulmonary toxicology of particles. I am a Fellow of the Institute of Biology, a Fellow of the Royal College of Pathologists and a Fellow of the Faculty of Occupational Medicine by research on particle toxicology. I hold the degrees of BSc (Hons – 1st Class) from the University of Stirling in Biology (1978), and a PhD (1982) and DSc (1998) from the University of Edinburgh. I have carried out 30 years of research into the harmful effects of inhaled particles including various types of naturally-occurring and manmade mineral fibres. I have published almost 300 papers and reviews and book chapters on particle toxicology, many of them concerning fibre toxicology. I have edited a major textbook on particle toxicology of the lung and am founding Editor in Chief of the Journal Particle and Fibre Toxicology. I have lectured extensively in undergraduate general toxicology and postgraduate toxicology specialising in lung toxicology of particles. I was employed by the Institute of Occupational Medicine as section head scientist from 1972-1992, by Napier University, Edinburgh as a Professor of Pathobiology 1992-2002, and the University of Edinburgh as Professor of Respiratory Toxicology from 2002 to the present. I sit and have sat on numerous UK Government, European and US committees that consider various strategic and regulatory aspects of the toxicology of inhaled particles and fibres.

Testing for genotoxicity *in vitro*

The mechanism of carcinogenicity caused by fibres is not fully understood, but at least two mechanisms may be operative [3]. Firstly, direct genotoxicity can be caused by fibres encountering cells, interacting with them, leading directly to DNA damage. This is mimicked in the assays which measure direct genotoxicity in which fibres are added directly to the target cells and genotoxic effects sought. Secondly, carcinogenesis can be driven by indirect effects through inflammation. In this case, the fibres affect the cells of the inflammatory system (the leukocytes) which increase in number and activity at the site of the fibre deposition. In this position the inflammatory cells release oxidants and mitogens and together these can very readily lead to mutation and proliferation, key processes in carcinogenesis. Indirect carcinogenesis would be assessed by determining the ability of the fibre to cause pro-inflammatory effects in target cells, on the assumption that the resulting inflammation would cause the genotoxicity. Both mechanisms of carcinogenesis are addressed in *in vitro* tests, where both direct genotoxic effects of fibres and pro-inflammatory effects of fibres can be demonstrated. As discussed below, however, these studies are flawed and liable to show false–positivity for long, non-biopersistent fibres in assays of both direct and indirect mechanisms of carcinogenicity as will be discussed below.

1) Direct genotoxicity A range of different assays are available for testing the *in vitro* genotoxicity of compounds and particles. These involve a range of different cell types, such as Chinese hamster V79 cells, Syrian hamster embryo cells, MET-5A (mesothelial) cells etc, and a range of endpoints including genetic damage as measured by formation of micronuclei, chromosomal aberrations, cell transformation, adduct formation, etc. These assays are considered to have some utility for the detection of the genotoxic effects of compounds of various types and, indeed, are included in "Other relevant data" in IARC monographs. They have also been used extensively for particles and fibres, including quartz, asbestos and, of special importance here, vitreous fibres. The role of biopersistence in the carcinogenicity of glass fibres is well understood and supported by numerous

animal studies and is extensively dealt with in the "Draft report on carcinogens: background document on glass wool fibres". These studies show that long biopersistent fibres are the effective dose for fibrosis and cancer in laboratory animals, while long non-biopersistent fibres have much reduced or no carcinogenic potential. The underlying mechanism is described to some extent in the document and concerns the fact that long biopersistent fibres can retain their structural integrity over the time required to migrate to the lung interstitium and the pleura. This leads to the induction of persistent inflammation, fibrosis and genotoxic effects in the lung itself, leading to fibrosis and carcinoma, whilst at the pleura these processes lead to fibrosis and mesothelioma. In contrast, long, non-biopersistent fibres undergo dissolution and breakage to harmless short fibres and so do not cause these effects to anything like the same extent.

Clearance half-time of mineral fibers as determined by the inhalation biopersistence protocol.

FIBRE NAME	FIBRE TYPE	WEIGHTED $T_{1/2}$ FIBRES L>20 μm (Days)
Fiber B	B01.9	2.4
Fiber A	Glasswool	3.5
Fiber C	Glasswool	4.1
Fiber G	Stonewool	5.4
MMVF34	HT stonewool	6
MMVF22	Slagwool	8
Fiber F	Stonewool	8.5
MMVF11	Glasswool	9
Fiber J	X607	9.8
MMVF 11	Glasswool	13
Fiber H	Stonewool	13
MMVF10	Glasswool	39
Fiber L	Stonewool	45
MMVF33	Special purpose glass	49
MMVF21	Stonewool	67
MMVF21	Stonewool	85
Amosite	Amphibole asbestos	418
Crocidolite	Amphibole asbestos	536

The time that a long fibre persists in the lungs , known as the retention half-time (or clearance half-time), varies between fibre types as shown in Table 1 [2] . Table 1 shows the retention/clearance half-times for the long fibre fraction of a number of vitreous fibre types and asbestos. It is clear that the half-times, that is the time for depletion to 50% of the long (>20 μm) fibres, ranges from 2.4 days to 85 days for various wools (data from Bernstein et al, 2007, Table 2). This is interpreted as meaning that between 2.4 and 85 days residence in the lung is required for half of the long fibres to be dissolved or, as is more likely, weakened

and broken into shorter fibres.

Table 2 shows the timescale of a range of in vitro tests of genotoxicity. As is evident from Table 2, the timescale of the average *in vitro* genotoxicity assay ranges from a few minutes to a few days. Clearly, even if the conditions in an *in vitro* cell culture genotoxicity assay were to mimic the conditions in the lungs that lead to dissolution and breakage of long fibres, the timescale of the longest *in vitro* assays is only one halftime of the fastest-dissolving fibres; for the great majority of wools the half-time greatly exceeds the length of an *in vitro* genotoxicity test. Therefore biopersistence, which is a key modifier of the carcinogenicity of non-biopersistent long fibres *in vivo,* cannot play a role in *in vitro* assay. This inevitably leads to false positives in *in vitro* genotoxicity for non-biopersistent fibres such as glass wool insulation.

Assay	**Time**	**Reference**
8-hydroxy-deoxy-guanosine	2 -20 hours	[4,5]
Morphological transformation of Syrian hamster embryo cells	7 days	[6]
Unscheduled DNA synthesis	1 day	[7]
Mutagenicity in A_L cells	6 days	[8]
Nuclear abnormalities in Chinese hamster ovary cells	2-3 days	[9]
Fibrin-induced hydroxyl radical formation	10 mins	[10]
Chromosomal aberrations	2 days	[11]
Induction of c-fos and c-gun proto-oncogenes	1-3 days	[12]

It is important to note that there are factors other than time that make *in vitro* assay unrepresentative of the events that occur in the lungs affecting biopersistence. The kinds of target cells that are appropriate for genotoxicity studies, such as epithelial cells and mesothelial cells *in vitro,* are exposed to fibres with a complete lack of defence in the form of macrophages or lung lining fluid in the cultures. The cells are exposed *in vitro* to large numbers of fibres under conditions which do not occur *in vivo,* where macrophages via the normal clearance mechanisms act to reduce the number of fibres that come in contact with epithelial cells and mesothelial cells. Additionally, the acid conditions inside macrophage phagolysosomes are far more extreme than would be encountered in epithelial cells or mesothelial cells which are not 'professional' phagocytes. Therefore the absence of macrophages from the *in vitro* system means that any dissolution that does occur will be much slower. In addition, in the lung the breathing movements are likely to encourage breakage of weakened fibres.

2) Indirect genotoxicity: assay of pro-inflammatory effects of fibres *in vitro*

The same argument about false positives in direct genotoxicity tests also pertains to *in vitro* assays demonstrating inflammatory effects in cultured cells. In this case, leucocytes or fixed cells are exposed to fibres *in vitro* for a few hours, (seldom more than one day) and then pro-inflammatory gene expression for cytokines or production or oxidants is measured. Long non-biopersistent fibres do not have the opportunity to undergo dissolution in the *in vitro* assays, and so are likely to produce false positives for pro-inflammatory effects. In these kinds of experiments, long fibres are more active than short fibres at producing pro-inflammatory effects in many studies [1,13], yet the fact that a non-biopersistent long fibre will undergo dissolution, break and become short or disappear entirely *in vivo* is not taken into account in assays that last only a few hours or days. Therefore, studies on the pro-inflammatory effects and oxidant-generating effects suffer from the same flawed interpretation as those where direct genotoxicity is being measured.

3) Other competent authorities

Other competent authorities have expressed similar concerns regarding the use of short-term assays that do not take account of biopersistence.

a) The IARC SVF Working group [17] reported that:-

'....4.3 Toxic effects in experimental systems
This section covers selected toxic effects of fibres in experimental systems that are believed to be potentially important in relation to the carcinogenic process. These endpoints include in-vivo effects such as inflammation and fibrosis, as well as selected in-vitro assessments including cytotoxicity, oxidant production and alterations to the cell cycle including proliferation and apoptosis. Genetic toxicology end-points are reviewed in section 4.5. It is important to appreciate the degree to which biopersistence plays a role in the different studies and end-points under review, as this property of fibres is thought to be critical in determining chronic toxicity and carcinogenic outcome in humans and in experimental animal systems. In-vitro assays are invariably short-term (i.e. from hours to days), and the effect of fibre durability is unlikely to be detected in such assays. [The Working Group noted that endotoxin is a potent environmental contaminant and its presence in fibre samples could enhance their ability to cause acute inflammation. The presence of endotoxin or the steps taken to inactivate it, were not always reported.] Therefore, short-term tests could give a misleading

impression of possible long-term biological effects. This will most likely become manifest as a false-positive result in an in-vitro assay for long, non-biopersistent fibres. For a non-biopersistent fibre, the effects seen in vitro may apply only to the time interval in vivo before the fibre begins to undergo dissolution or breakage. In contrast, a durable fibre may show the much more slowly and is more likely to give rise to pathological change....'

They also noted on page 337 in summarizing the genetic effects of man made vitreous fibers:

"A major gap in the current database is the absence of any studies that correlate genotoxic endpoints with the pathogenic effects of man made vitreous fibres in the same experimental animal system".

b) Another report was the product of the joint efforts of the members of an expert working group organized and convened by the International Life Sciences Institute Risk Science Institute [18].

'...Comment. There are several issues that limit the usefulness of in vitro tests for toxicity screening of fibers. For example,short-term in vitro assays of biological activity cannot allow for differences in biopersistence of fibers, and as a result, some nonbiopersistent fibers that are not pathogenic in vivo are positive in short-term in vitro tests (Hesterberg et al., 1983; Ye et al., 1999). In vitro cellular assays, in fact, have several technical limitations:
1. High doses of fibers are used to obtain a positive response; it is difficult to extrapolate from these high-dose, short-term exposures to low-dose, chronic exposures in vivo.
2. Fiber dose in cellular assays is often expressed in terms of mass of fibers rather than numbers of fibers, creating a major problem in relating in vitro to in vivo dose. In fact, number of long (>20 µm) fibers is a better dose metric to use in comparing potency between fiber types. Number of long fibers per cell is the optimal expression of dose, but number of long fibers per unit surface area of the culture dish is an acceptable alternative.
3. In vitro endpoints (e.g., release of inflammatory mediators, activation of transcription factors, induction of cell proliferation or apoptosis) are measured after a few hours or days, while in vivo responses to biopersistent fibers are sustained over several weeks or months. These endpoints have not been validated as screening assays that are predictive of long-term pathological effects in vivo.
4. The target cells used in short-term cellular assays are difficult to standardize and maintain. Stable primary cultures of mesothelial cells, alveolar cells, or terminal bronchial epithelial cells are not widely available. Cell lines used in the published in vitro studies have been derived from human lung tumors (e.g., A549 cells), spontaneously immortalized cells, or cells transfected with viral oncoproteins (e.g., Met5A) that may alter their genotoxic, apoptotic, or proliferative responses...'

c) The US Agency for Toxic Substances and Disease Registry (ATSDR) [19] has also stated:

"Notably absent are data on genotoxic end points following in vivo exposure of animal or humans to synthetic vitreous fibers. Results from short-term in vitro genotoxicity assays are of limited applicability to in vivo exposure scenarios because of evidence that long-term residence of synthetic vitreous fibers in the principal toxicity target, the lung, can lead to changes (dissolution, breakage into shorter fibers) that can decrease biological activities of longer fibers "
(see website http://www.atsdr.cdc.gov/toxprofiles/tp161.html)

4) Conclusion In summary, a number of authorities are in agreement with my own experience of the use of short-term *in vitro* assays for predicting genotoxicity of long non-biopersistent fibres in concluding that, in contrast to the usefulness of such short-term genotoxicity studies for many chemicals, their utility and the significance of reported findings are subject to a number of recognized limitations with regard to synthetic vitreous fibres.

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